

SUPPLEMENTAL MATERIAL

MDM2-mediated Ubiquitination of ACE2 Contributes to the Development of Pulmonary Arterial Hypertension

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Supplemental Methods

Supplemental Tables I-III

Supplemental Figures I-III

Supplemental Methods

Mouse lung EC isolation

Mouse lungs were perfused with 10 ml sterile PBS. The minced lung tissues were transferred to 25 mL 2mg/ml Type I collagenase (Worthington) and incubated at 37°C with gentle agitation for 45 min. Cell suspensions were filtered through 70 µm cell strainers (Thermo Fisher Scientific) after 12 trituration cycles. The cell suspension was further precipitated with anti-CD31 (Abcam) pre-conjugated Dynabeads (Invitrogen).

Supplemental Tables I-III

Supplemental Table I. Demographic and clinical data of patients from whom lung tissues and PAECs were obtained

Lung tissues			PAECs		
Patient ID	Sex	Age	Patient ID	Sex	Age
Control-1	Male	55	Control-1	Male	20
Control-2	Male	27	Control-2	Female	52
Control-3	Male	27	Control-3	Female	50
Control-4	Male	65	Control-4	Male	48
Control-5	Male	11	Control-5	Female	36
Control-6	Male	47	Control-6	Male	47
			Control-7	Male	49
			Control-8	Female	55
	Female 0%	38.7±20.3		Female 50%	44.6±11.4
IPAH-1	Female	30	IPAH-1	Female	27
IPAH-2	Female	33	IPAH-2	Male	13
IPAH-3	Female	28	IPAH-3	Male	40
IPAH-4	Female	46	IPAH-4	Female	32
IPAH-5	Female	19	IPAH-5	Female	57
IPAH-6	Female	25	IPAH-6	Male	53
			IPAH-7	Male	51
			IPAH-8	Female	16
	Female 100%	30.2±9.1		Female 50%	36.1±16.9

Supplemental Table II. The evidence mode of predicted E3 ligases

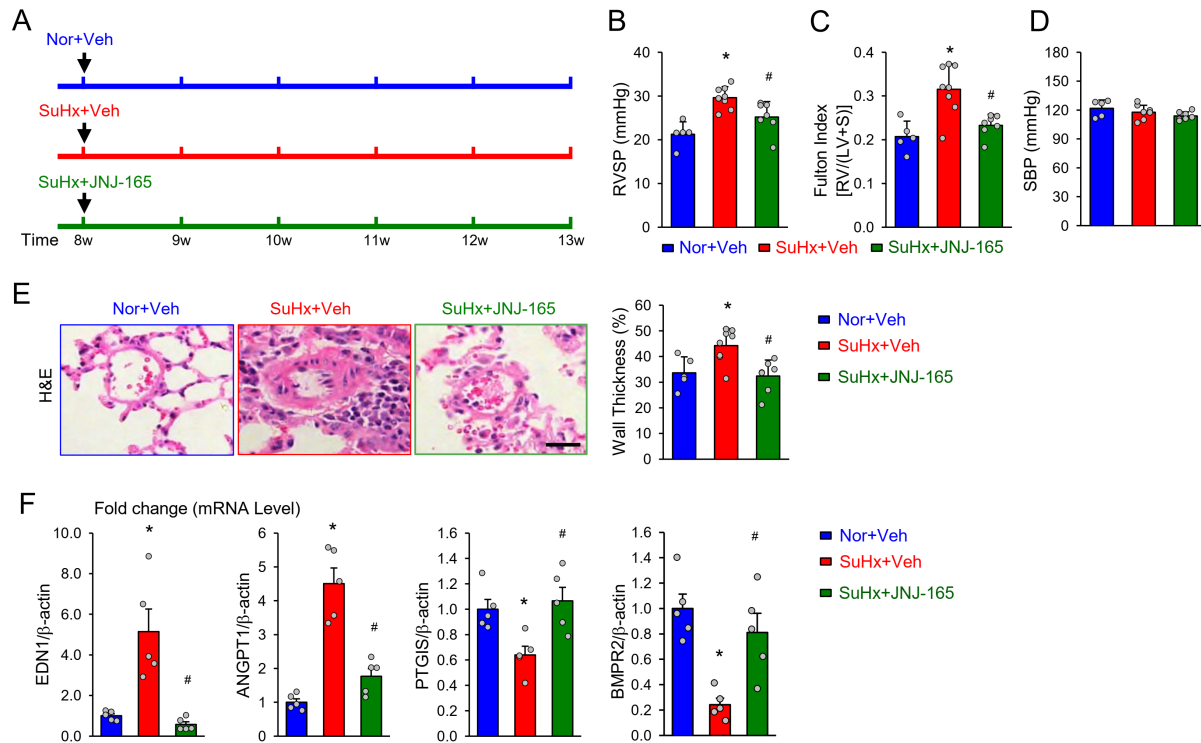
Rank	E3 ligase	RecName	Enriched domain pair	E3 recognizing motif	Network loops	Otholog interaction	Enriched GO pair	Confidence level	Score
1	MDM2	E3 ubiquitin-protein ligase MDM2		•	•		•	MIDDLE	0.716
2	NEDD4L	E3 ubiquitin-protein ligase NEDD4-like		•			•	MIDDLE	0.711
3	MIB1	E3 ubiquitin-protein ligase MIB1		•			•	MIDDLE	0.668
4	CBL	E3 ubiquitin-protein ligase CBL		•			•	LOW	0.653
5	SYVN1	E3 ubiquitin-protein ligase synoviolin		•			•	LOW	0.652
6	DTX1	E3 ubiquitin-protein ligase DTX1					•	LOW	0.647
7	CBLC	E3 ubiquitin-protein ligase CBL-C					•	LOW	0.647
8	SOCS7	Suppressor of cytokine signaling 7					•	LOW	0.647
9	CBX8	Chromobox protein homolog 8					•	LOW	0.646
10	RING1	E3 ubiquitin-protein ligase RING1					•	LOW	0.646

Supplemental Table III. The primers used for qPCR

Gene	Forward 5'–3'	Reverse 5'–3'
For human		
<i>β-actin</i>	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<i>MDM2</i>	GAATCATCGGACTCAGGTACATC	TCTGTCTCACTAATTGCTCTCCT
For mouse		
<i>β-actin</i>	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
<i>MDM2</i>	GGATCTTGACGATGGCGTAAG	AGGCTGTAATCTTCCGAGTCC
<i>EDN1</i>	GTGTCTACTTCTGCCACCTGGACAT	GGGCTCGCACTATATAAGGGATGAC
<i>BMPR2</i>	GTGCCCTGGCTGCTATGG	TGCCGCCTCCATCATGTT
<i>ANGPT1</i>	GGGGGAGGTTGGACAGTAA	CATCAGCTCAATCCTCAGC
<i>PGI2</i>	AAGATGGGAAACGGCTGAAG	CGAAGCCATATCTGCTGAGG
<i>VCAM-1</i>	AGTTGGGGATTTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
<i>VEGF</i>	CCAAGATCCGCAGACGTGTA	GACCCAAAGTGCTCCTCGAA
<i>PDGFA</i>	AGCATCCGGGACCTCCAG	AAGACCGCACGCACATTGG
<i>IL-6</i>	TGGAGTCACAGAAGGAGTGGCTAAG	TCTGACCACAGTGAGGAATGTCCAC
<i>MCP-1</i>	TTAAAAACCTGGATCGGAACC	GCATTAGCTTCAGATTACGG
<i>IL-1β</i>	ATGAGAGCATCCAGCTTCAA	TGAAGGAAAAGAAGGTGCTC
For rats		
<i>GAPDH</i>	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
<i>MDM2</i>	GGCGTAAGTGACCATTCTG	CCGATAGACCTCATCATCCT

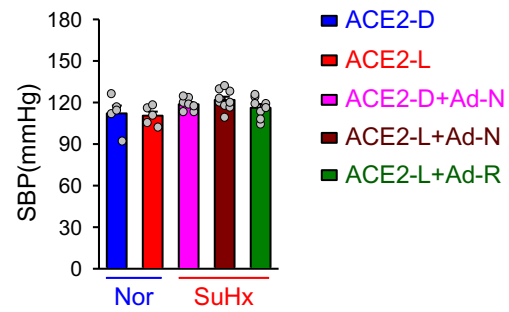
Supplemental Figures I-III

Supplemental Figure I



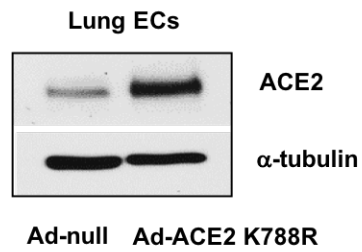
Supplemental Figure I. MDM2 inhibition prevents PH in mice. (A) Eight-week C57BL/6 mice were kept under hypoxia (10% O₂) with subcutaneous injection of SU5416 at 20 mg/kg once a week for 5 weeks. The SuHx mice were administered JNJ-165 (20 mg/kg/d) or vehicle at the beginning by oral gavage. The control group of mice were under normoxia for 5 weeks. (B-D) Right ventricular systolic pressure (RVSP) (B), Fulton index (RV/LV+septum [S] weight) (C) and systemic systolic blood pressure (SBP) (D) were measured. (E) HE staining of pulmonary arteries in lung sections (scale bar = 20 μ m). (F) qPCR analysis of the indicated mRNA in lung tissue of these mice. Data are mean \pm SEM (5-8 mice per group). Normally distributed data (B, C, D, E, and F) were analyzed by one-way ANOVA test between multiple groups. * $p < 0.05$ vs. Nor+Veh; # $p < 0.05$ vs. SuHx+Veh.

Supplemental Figure II



Supplemental Figure II. SBP in ACE2-S680D and S680L mice. Systemic systolic blood pressure (SBP) was measured in various groups of mice with treatment as indicated. Data are mean \pm SEM (5-9 mice per group). Normally distributed data were analyzed by one-way ANOVA test between multiple groups.

Supplemental Figure III



Supplemental Figure III. Increased ACE2 expression in lung ECs infected with Ad-ACE2-K788R. Ad-ACE2-K788R were delivered to C57BL/6 mice through intratracheal administration. Western blot analysis of ACE2 and α -tubulin in lung ECs isolated from mice 2 weeks after Ad-ACE2-K788R delivery.